Remarks

The foregoing amendment and the following remarks are believed merely to clarify Applicants' invention and do not raise new issues for consideration by the Examiner. Entry of this amendment after final rejection, which merely corrects typographical errors and a citation in the specification, and remarks is respectfully requested. Entry of the amendment and remarks will place the application in condition for allowance or materially reduce the issues for consideration on appeal. Thus, entry of the amendment and remarks is appropriate.

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendment, claims 25, 29-33, 36, 42 and 43 are pending in the application, with claims 25 and 36 being the independent claims. The changes to the specification are believed to introduce no new matter, and their entry is respectfully requested.

Based on the above amendment and the following remarks, Applicants respectfully request that the Examiner reconsider the outstanding rejection and that it be withdrawn.

Rejections under 35 U.S.C. § 112

The Examiner maintained her rejection of claims 25, 29-33, 36, 42 and 43 under 35 U.S.C. § 112, first paragraph, for alleged lack of enablement. According to the Examiner:

> [t]he specification is not enabling for the limitations of the claims wherein the recited composition of superoxide dismutase is delivered to neuronal cells or translocated into neuronal cells, or protects cells against oxidative damage.

(Office Action at 4).

Applicants respectfully traverse this rejection.

A specification is presumed to be enabling unless the Examiner provides acceptable objective evidence or sound scientific reasoning showing that it would require undue experimentation for one of ordinary skill in the art to make and use the claimed invention. *In re Marzocchi*, 439 F.2d 220, 224 (CCPA 1971) ("it is incumbent upon the Patent Office . . . to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement."); *see also In re Wright*, 999 F.2d 1557, 1562 (Fed. Cir. 1993) ("Without a reason to doubt the truth of the statements made in the patent application, the application must be considered enabling.").

The Examiner stated:

> The specification teaches a composition comprising superoxide dismutase (SOD) attached to a fragment of a Clostridium toxin, for the purpose of translocating the SOD into neuronal cells and thus protecting them from oxidative damage. However, the disclosure is not enabling for use of the composition to translocate SOD into neuronal cells and reduce oxidative damage. Experiments are described in which the SOD composition is applied to a culture of NG-108 neuroblastoma cells both with and without the superoxide generator duroquinone (Figure 5). Measurements were made that Applicants contend demonstrate protective effects on the cells against superoxide-induced oxidative stress. However, the methods were not described in sufficient detail to enable one skilled in the art to determine the protective effects of the SOD composition on oxidative stress in neuronal cells in the manner described. It is not known, for example, and not disclosed in the Specification, how

> absorbance of light at 570 nm is related to oxidative stress. No experiments were performed demonstrating that the SOD/Clostridium composition was translocated into the cells. No evidence was presented that the cells were oxidatively stressed or damaged. Furthermore, the treatment groups seem indistinguishable from each other and there appears to be no concentration effect of superoxide dismutase on the measured variable-the SOD/Clostridium composition had approximately the same effect at zero concentration as the effect at a concentration of 100.

(Office Action at 5).

As noted above, a specification is presumed to be enabling unless the Examiner provides evidence or sound scientific reasoning to doubt the enablement of an invention. The Examiner's questioning of the sufficiency of disclosure of the present specification is not a substitute for such evidence or reasoning.

In any event, the specification does in fact demonstrate that the claimed composition reduces oxidative stress in neuronal cells. Applicants respectfully direct the attention of the Examiner to Example 10 wherein the neuroblastoma cell line NG108-15 was treated with MnSOD conjugated to purified heavy chain of botulinum neurotoxin serotype A (BoNT/A HC). The conjugate was replaced with duroquinone, a compound known to induce oxidative stress. See the abstract of Wilde *et al., J. Neurochem.* *69*:883-6 (1997), attached hereto as Exhibit A, and the abstract of Musser and Oseroff, *Photochem. Photobiol.* *59*:621-6 (1994), attached hereto as Exhibit B. The media was then removed and replaced with the dye MTT which is reduced to formazan dye crystals. The extent of reduction of MTT is a measure of mitochondrial activity and, thus, a measure of the oxidative stress to the mitochondria. See Liu *et al., J. Cell. Mol. Med.* *7*:49-56 (2003), attached hereto as Exhibit C. As shown at the top of page 51 of Liu *et*

al., absorbance at 570 nm is used as a measurement of formazan dye production. Thus, absorbance at 570 nm is directly related to the level of mitochondrial activity.

The results are shown in Figure 5. NG108 cells that were treated with no duroquinone (▲) showed the largest absorbance (about 0.800), indicating that such cells had the highest level of mitochondrial activity. Cells treated with just duroquinone (□) had a reduced level of absorbance (about 0.640) compared to cells treated with HC/A MnSOD + 56 mM KCl (♦). With increasing concentrations of conjugate, the absorbance increased from about 0.760 to about 0.800 (the absorbance of cells not treated at all with duroquinone). These results demonstrate a dose-dependent relationship between levels of mitochondrial activity and concentration of conjugate. Moreover, cells treated with the highest concentrations of conjugate were protected from the stress-inducing effects of duroquinone.

While the presence of the construct alone, in the absence of KCl, did not protect the cells from the effect of duroquinone (■), this is to be expected when testing *in vitro*. The toxin appears to be internalized along with receptors that are internalized when the nerves are firing. The effect of KCl is to simulate, *in vitro*, the effect of the nerve firing by depolarizing the membrane in a similar manner to an electrical impulse arriving at the terminal *in vivo*. Thus, *in vitro*, KCl helps the receptor to turn over and internalize the heavy chain construct. *In vivo*, addition of potassium ions is not necessary for function of the conjugate, as the nerve cells are firing in an alive animal.

With regard to the Examiner's statement that no experiments were performed demonstrating that the SOD/Clostridium composition was translocated into the cells, Applicants note that for any change in mitochondrial function to take place, the

composition of the invention must be internalized into the cell. Furthermore, the results of the experiments demonstrate that the oxidative stress induced by duroquinone is indeed reduced by the conjugate. The success in demonstrating reduction in oxidative stress in the presence of the conjugate is compelling evidence that in fact the conjugate is translocated into the cell.

Applicants remind the Examiner that the enablement requirement of 35 U.S.C. § 112, first paragraph, is satisfied if the claimed invention is enabled so that any person skilled in the art can make and use the invention without undue experimentation. *See In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). In order to establish a *prima facie* case of non-enablement, the Examiner has the initial burden to set forth a reasonable basis to question the enablement provided for the claimed invention. *See In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). Applicants submit that the claimed composition can be made and used by those of ordinary skill in the art without undue experimentation. Additionally, Applicants respectfully assert that the Examiner has not provided acceptable evidence or sound scientific reasoning as to why the specification does not enable the claimed invention. Hence, a *prima facie* case of non-enablement has not been established.

Reconsideration and withdrawal of the rejection under 35 U.S.C. § 112, first paragraph, therefore are respectfully requested.

Conclusion

All of the stated grounds of rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the

Examiner reconsider the presently outstanding rejection and that it be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

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Differential vulnerability of the CA1 and CA3 subfields of the hippocampus to superoxide and hydroxyl radicals in vitro.

Wilde GJ, Pringle AK, Wright P, Iannotti F.

J Neurochem. 1997 Aug;69(2):883-6.

Department of Clinical Neurological Sciences, University of Southampton, Southampton General Hospital, England, U.K.

The relative roles of the superoxide and hydroxyl radicals in oxidative stress-induced neuronal damage were investigated using organotypic hippocampal slice cultures. Cultures exposed to 100 microM duroquinone, a superoxide-generating compound, for 3 h developed CA1-selective lesions over a period of 24 h. The damage accounted fo approximately 64% of the CA1 subfield, whereas CA3 showed just 6% damage, a pattern of damage comparable to that observed following hypoxia/ischaemia. Duroquinone-induced damage was attenuated by a spin-trap agent. In contrast, hydroxyl radical-mediated damage, generated by exposure to 30 microM ferrous sulphate for 1 h, resulted in a CA3-dominant lesion. The damage developed over 24 h, similar tc that observed with duroquinone, but with approximately 45% damage in CA3 compared with only 7% in CA1. These data demonstrate a selective vulnerability of the CA1 pyramidal neurones to superoxide-induced damage and suggest that of the free radicals generated following hypoxia/ischaemia, superoxide, rather than hydroxyl radical, is instrumental in producing neuronal damage.

Publication Types:
- Journal Article

MeSH Terms:
- Animals
- Benzenesulfonates/pharmacology
- Benzoquinones/pharmacology
- Brain Ischemia
- Cell Death
- Ferrous Compounds/pharmacology

1: Photochem Photobiol. 1994 Jun;59(6):621-6. Related Articles, Links

The use of tetrazolium salts to determine sites of damage to the mitochondrial electron transport chain in intact cells following in vitro photodynamic therapy with Photofrin II.

Musser DA, Oseroff AR.

Department of Dermatology, Roswell Park Cancer Institute, Buffalo, NY 14263.

A method is described utilizing the tetrazolium salts neotetrazolium chloride (NTC), triphenyltetrazolium chloride (TTC), C,N-diphenyl-N'-4,5-dimethylthiazol-2-yltetrazolium bromide (MTT) and various substrates to elucidate damage to the mitochondrial electron transport chain of intact cells following in vitro photodynamic therapy (PDT). Using this methodology, a portion of the dark toxicity manifested by Photofrin II (PII) was found to occur prior to entry of electrons into the transport chain through Complex I, as evidenced by the fact that the inhibition of MTT reduction was reversible by the addition of malic acid to the culture media. A second site of dark toxicity was found to be Complex IV (cytochrome oxidase). After photoirradiation of the cells, Complex I was found to be affected since malic acid could no longer reverse the inhibition of MTT reduction but it could be reversed by the addition of succinic acid, whose electrons enter the transport chain at Complex II. A second and more sensitive site of photoirradiation damage was found to be Complex IV. A region near cytochrome C was also affected by photoirradiation but appreciably less so than noted for Complexes I and IV. A kinetic analysis of MTT and TTC reduction following photoirradiation indicated that MTT reduction was sustained at a normal rate for 1 h after which it slowed down and eventually plateaued. In contrast, TTC reduction was found to be inhibited almost immediately indicating Complex IV is extremely susceptible to photoirradiation damage. Compared to other assays of mitochondrial function requiring subcellular fractionation, the use of tetrazolium salts is simpler to perform and can be done using physiologically relevant conditions.

PMID: 8066121 [PubMed - indexed for MEDLINE]

J.Cell.Mol.Med. Vol 7, No 1, 2003 pp. 49-56

Glucose deprivation induces mitochondrial dysfunction and oxidative stress in PC12 cell line

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Abstract

Glucose metabolism plays a pivotal role in many physiological and pathological conditions. To investigate the effect of hypoglycemia (obtained by glucose deprivation) on PC12 cell line, we analyzed the cell viability, mitochondrial function (assessed by MTT reduction, cellular ATP level, mitochondrial transmembrane potential), and the level of reactive oxygen species (ROS) after glucose deprivation (GD). Upon exposure to GD, ROS level increased and MTT reduction decreased immediately, intracellular ATP level increased in the first 3 hours, followed by progressive decrease till the end of GD treatment, and the mitochondrial transmembrane potential ($\Delta\Psi_m$) dropped after 6 hours. Both necrosis and apoptosis occurred apparently after 24 hours which was determined by nuclei staining with propidium iodide(PI) and Hoechst 33342. These data suggested that cytotoxity of GD is mainly due to ROS accumulation and ATP depletion in PC12 cells.

Keywords: glucose deprivation • ATP depletion • mitochondrial membrane potential • reactive oxygen species • apoptosis • necrosis

Introduction

The pathogenesis of brain damage under conditions of metabolic stress continues to be of great interest, since much still needs to be understood. Many *in vivo* and *in vitro* models were established to mimic the ischemia-like pathological process. Rat pheochromocytoma (PC12) cells, originated from the adrenal medulla, synthesize and release catecholamines, is a well known neuronal model for *in vitro* ischemic studies [1]. Ischemic injury model of PC12 cells (oxygen and glucose deprivation, OGD) has been well established to investigate the mechanism of ischemia-induced cell death, and this model is also widely used for the development of neuroprotective drugs against ischemic insults [2]. However, OGD is a disastrous stimulus, and usually leads to rapid cell death therefore it is difficult to determine the molecular mechanisms of OGD

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induced cell injury which involved vigorous and complicated cellular changes. An alternative approach is to investigate the effect of hypoglycemia and hypoxia respectively. Glucose metabolism is the key point in maintaining the balance between the life and death, and glucose serves as an important regulatory factor in the ischemic injury cascade [3].

The cell death scenarios linked to glucose metabolic inhibition include ATP depletion and oxidative stress which are tightly associated with the mitochodrial function. Glucose deprivation (GD) results in ATP depletion and this in turn triggers the death cascade. Severe ATP depletion usually leads to necrosis since ATP is required for many steps in apoptosis. Glucose deprivation also results in oxidative stress and the alteration of the redox status of cells triggers stress-activated or other signal transduction pathways resulting in cell death [4–6]. In recent years, many reports showed that the loss of mitochondrial potential and the dysregulation of Ca^{2+} are also involved in this process [7–8]. Some studies have elucidated the cytotoxity of glucose deprivation in PC12 cells, but there is no systematic and detailed description for this process yet [9-10]. Therefore, our work presented here is focused on the dynamic changes of mitochondrial function in PC12 cells undergoing glucose deprivation.

Material and methods

Cell Culture

PC12 cells, purchased from American Type Culture Collection (ATCC), were grown routinely in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated horse serum (GibcoBRL), 5% fetal calf serum (GibcoBRL), 50 U/ml penicillin and 100μg/ml streptomycin, maintained at 37°C in a humidified incubator containing 95% air and 5% CO_2. The cells were fed every two days and passed twice a week. Cells were plated at 100,000 cells/cm^2 on poly-L-lysine-coated (100μg/ml) 96-well plates (0.1 ml/well) for MTT assay and DCF fluorescence assay, 24-well plates (0.5 ml/well) for Hoechst 33258 staining and ATP measurement, and on 12-well plates (1 ml/well) for mitochondrial potential assay.

Glucose deprivation

The cells were gently washed three times with phosphate-buffered saline and then incubated in glucose-free DMEM medium containing 2% horse serum and 1% fetal calf serum for indicated further time. The control cells were treated in the same way while incubated in high-glucose DMEM medium containing 1% horse serum and 1% fetal calf serum.

Trypan blue exclusion

The culture medium was removed and replaced by 0.1% trypan blue solution in phosphate-buffered saline for 3 min at room temperature. Viable cells in 10 randomly chosen fields were counted at 200-fold magnification using bright-field and phase-contrast microscopy for blue cells and total cells respectively.

Fluorescent staining of nucleus

Apoptosis and necrosis were distinguished using combined staining of chromatin dye, Hoechst dye 33342 and propidium iodide (PI) (Molecular Probes) [11]. Hoechst 33342 (λ_{ex} 360nm, λ_{em}490nm) freely enters living cells and therefore stains the nuclei of viable cells, as well as those that died by apoptosis or necrosis. Apoptotic cells can be distinguished from viable and necrotic cells on the basis of nuclear condensation and fragmentation, PI(λ_{ex} 536nm, λ_{em} 620nm) enters only cells with damaged cell membranes, then viable cells are PI negative while necrotic cells are PI positive. In brief, at the end of the cell culture, Hoechst was added to the culture medium at 1μg/ml for 10 min, and cells were incubated with PI at 1μg/ml for 10 min.

The nuclei were counted according to nucleus morphology and label.

MTT reduction assay

The determination of the mitochondrial function was performed by measuring the MTT reduction ability of PC12 cells, according to the method of Mosmann [12]. MTT, which reacts with dehydrogenases and cofactors of the respiratory chain, is an indicator of mitochondrial activity [13]. In brief, MTT was dissolved in PBS at 5 mg/ml and was added to culture medium at the end of incubated time, the final concentration was 0.5mg/ml.

After an additional 3-hr incubation at 37°C, 0.1 ml isopropanol/HCl was added to each well, and the absorbance at 570 nm, of solubilized MTT formazan products, was measured. Results were expressed as the percentage (%) of MTT reduction, assuming the absorbance of control cells as 100%.

ATP quantification

Intracellular ATP content was determined based luciferin/luciferase method [14] with the use of ATP bioluminescent assay kit (Boehringer). The cells supplemented with glucose or in hypoglycemic conditions, were washed with cold phosphate-buffered saline and lysed with 100 µl of cell lysing buffer. Then, 1 µl of the lysate was diluted to 100 µl with water and mixed with 100 µl of luciferase/luciferin reagent. After 10 s, the light emitted was recorded using a luminometer at 562 nm and integrated over 5s.

Protein quantification

Protein concentrations were determined by the method of Bradford [15], with bovine serum albumin as standard.

Measurement of mitochondrial transmembrane potential with DiOC6(3)

Mitochondrial transmembrane potential of PC12 cells during hypoglycemic treatment was studied with 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) (λex max 484 nm, λem max 501 nm) (MolecularProbes) [16]. In living cells, this cyanine dye can accumulate in the mitochondrial matrix under the influence of the mitochondrial transmembrane potential. PC12 cells were collected and resuspended with culture medium to 10^6 cells/ml, and incubate at 37°C for further 15min, then $DiOC_6(3)$ fluorescence was immediately recorded with flow cytometry (Becton Dickinson). For each sample, 10,000 cells were acquired for data analysis.

DCF fluorescence measurement

In order to measure the ROS production in PC12 cells under glucose deprivation, we used the DCFH-DA method as described previously [17]. DCFH-DA is membrane permeable, and it can be enzymatically converted to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS. At the end of the hypoglycemic treatment, PC12 cells (10^4 cells per well in a 96-well plate) were loaded with DCFH-DA(final concentration 100µM) for 30 min, fluorescence was monitored on a Cyto Fluor Multi-Well Plate Reader with λ_{ex} 485 nm and λ_{em} 530 nm. The results were expressed as a relative percent of DCF-fluorescence in control cells.

Data Analysis

Data were expressed as means±SEM from at least three independent experiments. Statistical significance analysis was determined by using the Student's *t*-test or analysis of variance (ANOVA; *P* value, 0.05 was considered significant).

Results

Glucose deprivation induced cell death

Previous studies have demonstrated that glucose deprivation can induce apoptosis in PC12 cells. We therefore initiated a series of experiments to investigate the effect of glucose deprivation treatment. Cell viability was determined with trypan blue exclusion assay. Apoptotic or necrotic cells were assessed by the nuclei staining with Hoechst 33342 and PI. After the treatment of glucose deprivation for 24 hours, the percentage of trypan blue positive cells was 19±4.2% (values of the percent survival for three individual cultures), and after 48 hours, the percentage of positive stained cells increased to 69±3.1%, while the cells of control group are almost all negative for trypan blue. Hoechst 33258 and PI stained cells were visualized by fluorescence microscopy. As Figure 1 shows, the cells with condensed chromatin and fragmented nuclear DNA were considered as apoptotic. The percentage of viable cells, apoptotic cells and necrotic cells in each culture was determined. At least 200 cells were counted in each experiment. Upon the exposure to GD for 24 hours, apoptotic cells were 7.8±2.2%, necrotic cell were 8.5±1.9%. After GD treatment for 48 hours, apoptotic cells were 21.6±3.1%, necrotic cells were 59.5±12.8%. In

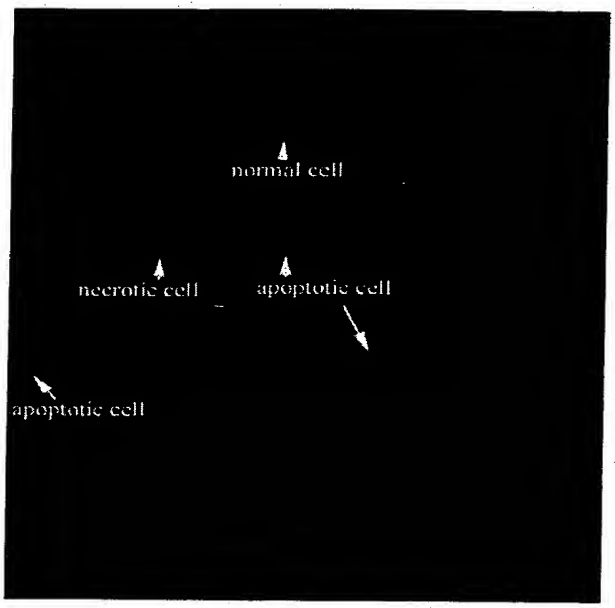

Fig. 1 Representative confocal fluorescence microscopy of PC12 cells stained with H-33342 and PI after GD treatment for 24 hours (x400), cells were grouped as below, (a) normal cells: showed normal morphology of nuclei and negative stained with PI. (b) necrotic cells: showed normal morphology of nuclei and positive stained with PI. (c) apoptotic cells: showed condensation and fragmentation of nuclei, including both PI positive and negative stained cells, nuclei of these cells were condensed and fragmented, demonstrating that apoptosis was primary cause of cell death.

control cells, both necrotic and apoptotic cells were lower than 1%.

Glucose deprivation decreased the MTT reduction in PC12 cells

The capacity of MTT reduction is an index of mitochondrial function, data presented in Figure 2 indicated that the MTT reduction decreased immediately and rapidly at the beginning of glucose deprivation, after 3 hours, it decreased slowly to a level which was about half of the control cells at the time of 24 hours, thereafter, an obvious decrease appeared again, and at the end of GD treatment (48 hours), the MTT reduction ability was less than 20% of control cells. Because the loss of MTT reduction was due to mitochondria dysfunction, then MTT was also an index of cell viability, and the data presented here was in accordance with the cell survival assay.

Effect of glucose deprivation on intracellular ATP level of PC12 cells

Glucose is the main substrate for intracellular ATP generation; PC12 cells are neoplastic in nature and they have a high rate of glycolysis accompanied by a large production of lactate and a low use of glucose through the Krebs cycle [18]. Data presented in Figure 3 shows that, after the first 3 hours of GD, ATP content was increased to a higher level than

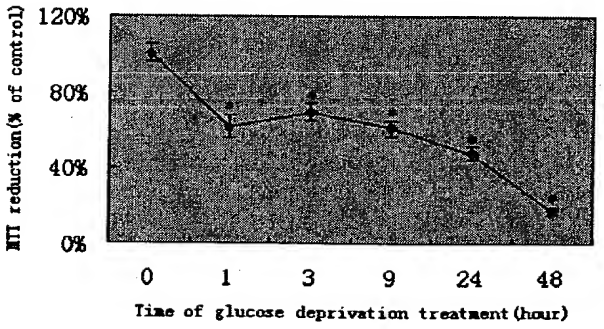

Fig. 2 Time course of the effect of glucose deprivation on PC12 cell MTT reduction. Data, expressed as the percentage (%) of control values, are means ± SEM (n=6) of a representative experiments. **P* <0.01 compared to control cells.

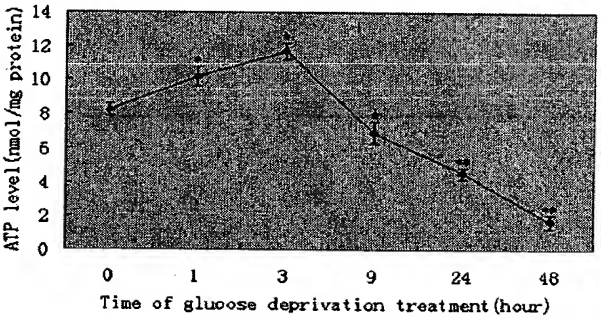

Fig. 3 Time course of the effect of glucose deprivation on intracellular ATP content of PC12 cells. The values expressed as the percentage (%) of control cells, are means ± SEM (n=6) of a representative experiments. **P* <0.05, ** *P* <0.01 compared to control cells.

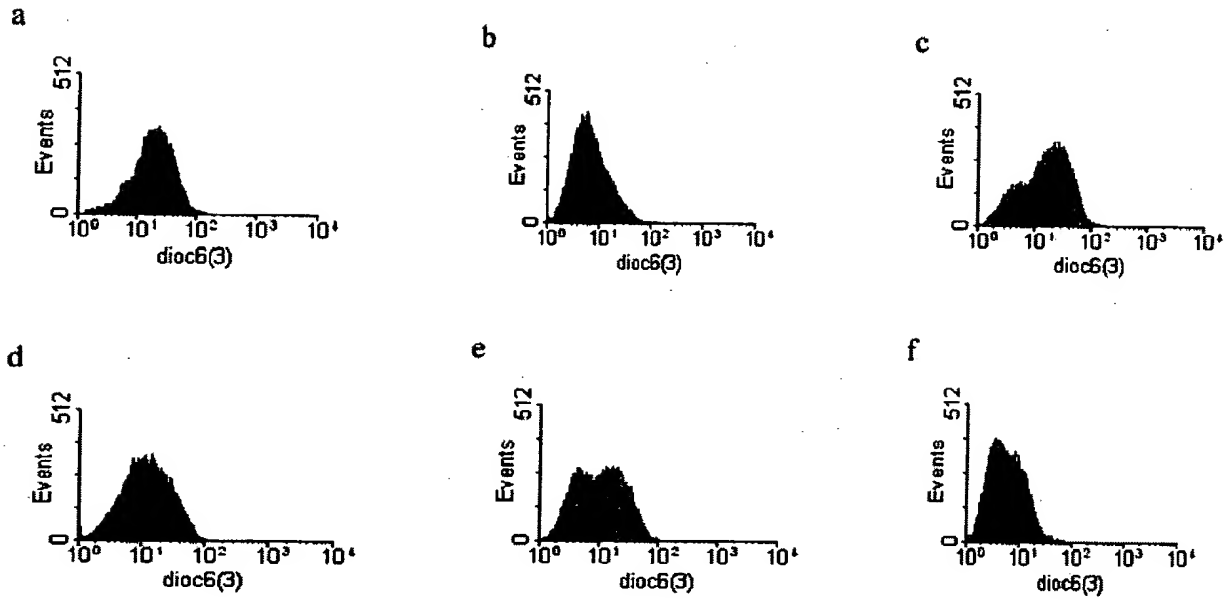

Fig. 4 Effect of glucose deprivation on the mitochondrial transmembrane potential($\Delta\Psi$m) of PC12 cells.(a) control cells (b) cells treated with 100μM carbonyl cyanide chlorophenylhydrazone(CCCP) to fully depolarize mitochondria (c) glucose treatment for 3 hours (d) glucose treatment for 6 hours (e) glucose treatment for 24 hours (f) glucose treatment for 48 hours.

control cells and afterwards progressively decreased during the rest time. Previous studies reported that, after glucose depletion, other energy sources, such as amino acids and glutamine in culture medium, could be used to generate ATP. The inhibition of glycolysis may stimulate mitochondria to use substrates other than glucose to maintain ATP level. Thus, an increase of ATP content was observed in the early stage of GD treatment, and even in the condition of hypoglycemia for 24 hours, ATP content was kept at 50% of control cells.

Effect of glucose deprivation on mitochondrial transmembrane potential ($\Delta\Psi_m$)

Recently, mitochondria has been regarded as the key regulator of cell death [19]. Healthy mitochondria maintain a negative membrane potential across the mitochondrial inner membrane, collapse of $\Delta\Psi_m$ lead to a reduction of mitochondrial mass which could activate the cell death cascade in turn. $DiOC_6$ (3), a fluorescent dye that incorporate into mitochondria in a $\Delta\Psi_m$ dependent manner was used to evaluate the changes of mitochondrial potential during GD treatment. As Figure 4 showed, in the first 3 hours, the mitochondrial potential was maintained well, the decrease of $\Delta\Psi_m$ became obvious only after 6 hours. Nearly half of cells were depolarized after 24 hours of GD and at the end of GD period (48 hours), cells were totally depolarized similarly to the cells treated with

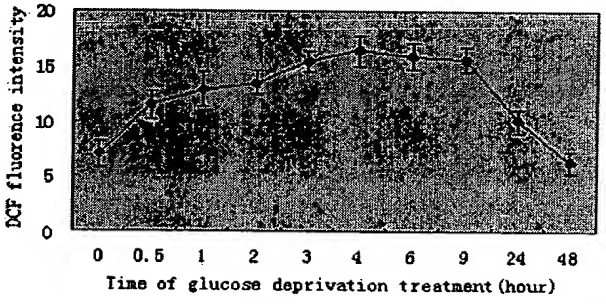

Fig. 5 Time course of the effect of glucose deprivation on ROS accumulation of PC12 cells. The values presented are means ± SEM (n=6) of a representative experiments.

carbonyl cyanide chlorophenylhydrazone (CCCP) to fully depolarize mitochondria.

Glucose deprivation induced a rapid increase of ROS in PC12 cells

It is well known that the mitochondria are the site of pro-oxidant production, and ROS are by-products of oxidative phosphorylation, and it has been suggested that mitochondria are the main source of ROS after cell damage. In our study, DCFH-DA method was used to monitor the ROS level during glucose deprivation insult. Our data indicated that the accumulation of ROS was a quick response to the GD treatment. ROS level increased nearly 2 fold within 30 min, and increased steadily to the peak at the time of 4 hours for GD, after which it followed a progressive decline to normal ROS level.

Discussion

In recent years, the pathogenesis of brain damage under conditions of impaired energy metabolism has been extensively investigated. PC12 cells oxygen-glucose deprivation is a well established and widely used *in vitro* model for ischemic studies, while the investigations for glucose deprivation alone in PC12 cells are less used than the OGD system due to its limitation in modeling ischemia. In our point, the elucidation of the cellular and molecular process in PC12 cells undergoing glucose deprivation will contribute to understand the pathogenesis of ischemic injury. Furthermore, some neuronal degenerative diseases such as Alzheimer's disease and amyotrophic lateral sclerosis (ALS) [20-22] seen to associate with the glucose deprivation induced insult and it has been reported that amyloid β-peptide (Aβ) inhibits glucose transport in neurons by a mechanism involving oxidative injury of the neuronal glucose transporter, GLUT3. Therefore, the decrease of glucose transport may trigger a cell death cascade similar to the process occurred in glucose deprivation models.

Cell damages induced by glucose deprivation depend greatly on the cell type and the method of GD treatment. We have reported the cytotoxical effect of GD on CHL cells which were much more susceptible than PC12 cells upon glucose deprivation [23]. Our unpublished data showed that PC12 cells could sustain growth in glucose-free medium (containing 10% serum) for more than 60 hours even in the presence of 2-deoxyglucose (glycolysis inhibitor) cell damage became obvious only at after the serum concentrations lower than 2%. PC12 cells were reported to undergo apoptosis upon the GD treatment, our data demonstrated that both apoptotic and necrotic cell death were involved in this insult, and the necrosis was the predominant form of cell death in our system. This phenomenon is consistent with the ischemia-reperfusion induced insult *in vivo* which is also composed of necrosis and apoptosis [24]. For an individual cell undergoing glucose deprivation, the choice between apoptosis and necrosis is settled by apoptogenic factors which can or cannot act before the bioenergetic catastrophe disrupt the plasma membrane integrity.

However, it can be debated about what triggered the cell death cascade in response to hypoglycemic stress. When cellular ATP stores were severely depleted to a level incompatible with maintenance of basal metabolism and activity of membrane transport pumps, cells were deemed to die due to the energetic catastrophy. In our study, cell death occurred even though intracellular ATP content was maintained at more than 50% of control cells. Thus, there might be other initiator of cell death, possibly ROS levels. Glucose metabolism results in the formation of not only ATP but also the redox potential NADPH. Therefore, in addition to its well known role in energy production, glucose metabolism appears to be related to the metabolic detoxification of intracellular hydroperoxides formed as byproducts of oxidative metabolism in mitochondria.

It was reported that glucose deprivation led to accumulation of pro-oxidants, presumably superoxide and hydrogen peroxide, as a result of the metabolic shift to oxidative phosphorylation, and the glucose deprivation-induced oxidative stress has been shown to activate signal transduction pathways leading to apoptosis in a breast carcinoma cell line (MCF-7/ADR) [25]. Our results also demonstrated that ROS accumulated immediately after the onset of glucose deprivation which was followed by the decreased MTT reduction ability of mitochondria. This phenomenon indicated a failure in elimination of oxidants which is probably due to inadequate production of NADPH via the pentose phos-

phate pathway. After 4 hours for GD treatment, ROS level reached the highest point, then declined afterwards to the normal level. This might be due to two reasons. First, the increased level of ROS would stimulate the cellular defensive system such as glutathione/glutathione and peroxidase/glutathione reductase system to decrease the intracellular hydroperoxide level. Second, the further depletion of substrate of TCA cycle such as amino acids would inhibit the oxidative phosphorylation, and in turn block the ROS accumulation.

Both oxidative stress and ATP depletion were reported to be involved in the dysfunction of mitochondria which was marked with the collapse of mitochondrial transmembrane potential($\Delta\Psi_m$), and the drops of $\Delta\Psi_m$ induced Ca^{2+} release, increased production of ROS, and precipitated release of proapoptotic factors such as cytochrome *c* from mitochondria. Therefore, we detected the alteration of $\Delta\Psi_m$ during GD treatment. The changes of $\Delta\Psi_m$ upon GD is controversial because many groups have observed an early increase in $\Delta\Psi_m$ followed by a further decline. Our data showed that there was no apparent alteration of $\Delta\Psi_m$ during the first 3 hours, and the decline of $\Delta\Psi_m$ became obvious after 6 hours.

In summary, glucose deprivation induced cytotoxity and mitochondria dysfunction mediated by oxidative stress and by ATP depletion. Our present study was focused on the dynamic changes of mitochondria function, while the relationship between these phenomena and the underlying molecular mechanism needs further investigation.

Acknowledgments

The present work was supported by National Natural Foundation of China (39670230).

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